Hirano Body Filaments Contain Actin and Actin-Associated Proteins

PAMELA G. GALLOWAY, M.D., GEORGE PERRY, PH.D., AND PIERLUIGI GAMBETTI, M.D.

Abstract. Hirano bodies are eosinophilic, rod-shaped intraneuronal inclusions whose frequency increases with age and with Alzheimer's disease. To investigate their composition and possible relationship to the neuronal cytoskeleton, we employed immunocytochemistry and immunoelectronmicroscopy by using antisera to cytoskeletal proteins. The presence of actin, α-actinin, vinculin and tropomyosin was demonstrated diffusely throughout the Hirano body. The presence of these proteins supports the contention that Hirano bodies are derived from an abnormal organization of the neuronal cytoskeleton. The staining of Hirano bodies with fluorescent labelled phalloidin, a probe with a unique affinity for F-actin, indicates that the actin in Hirano bodies is in the F-state. Results of high voltage electron microscopy on 1.0 and 0.5 micron sections confirm the purely filamentous nature of Hirano bodies. These findings suggest that the mechanism of formation of Hirano bodies is different from that of the neurofibrillary tangle, another characteristic intraneuronal inclusion seen in Alzheimer patients.

Key Words: Actin; Aging, central nervous system; α-actinin; Alzheimer's disease; Cytoskeleton; Hirano bodies; Tropomyosin.

INTRODUCTION

The neuronal cytoskeleton is a complex and pervasive cell structure (1, 2). During its lifespan, it goes through a series of stages which include synthesis, assembly, post-translational modification, transport and breakdown. Impairment of any of these stages will affect subsequent ones, causing a cascade of events which may result in the formation of abnormal inclusions. Thus, it is not surprising that the cytoskeleton is involved in a variety of pathological conditions affecting neurons.

Alzheimer's and Pick's diseases and progressive supranuclear palsy are characterized by the presence of inclusions which contain components of the cytoskeleton (3–6). The Hirano body is another neuronal inclusion which is commonly present in neurons of non-demented old individuals, and in increased numbers in patients with Alzheimer's and other diseases (7–9). It is most commonly seen in neurons of the Sommer sector of the hippocampus (10) and in the underlying stratum lacunosum (11), but rarely is it located in the spinal cord (12), cerebellum (13), cerebral cortex (14), Schwann cells (15) and oligodendroglia (16). Hirano bodies also occur in experimental animals (17, 18). With electron microscopic examination, Hirano bodies show a paracrystalline structure made up of sheets of 6–10 nm parallel filaments. The filaments of adjacent sheets are superimposed at oblique angles (10–14). Another interpretation of their composition is that of granular ribosomal material arranged on filaments (22).

The chemical composition of Hirano bodies is not known. An immunocytochemi-
ical study has demonstrated the presence of epitopes of actin (11), the major component of microfilaments (23). The presence of actin suggests that Hirano bodies may result from an alteration of the cytoskeleton.

Actin is present throughout the cell either as a soluble globular monomer (G-actin) or as polymers (F-actin) which are called microfilaments and are 4–6 nm in diameter (23, 24). The microfilaments in neurons, as in other cells, are organized in a system that is dispersed throughout the cell (24). They are highly dynamic; the filaments rapidly assemble and disassemble providing a quick response to functional requirements of the cell (24). Control over this dynamic state is facilitated by actin-associated proteins (25–27). These proteins are classified according to their function (27) as cross-linking proteins, proteins which cap filament ends and regulate filament length (many also sever actin filaments) and those which bind to G-actin and stabilize the monomer pool. All these proteins function by binding to the appropriate form of actin.

To study the composition of Hirano bodies, we performed high voltage electron microscopy on thick sections to resolve whether Hirano bodies consist of superimposed filaments or granular structures arranged on filaments. We used ultrastructural immunocytochemistry to determine whether actin epitopes are uniformly present in all filaments in Hirano bodies, or are preferentially located in certain filament populations. We examined whether G-actin or F-actin is present in the Hirano bodies using phalloidin, a marker for F-actin (28). Moreover, we carried out a light and electron microscopic immunocytochemical study to look for actin-associated proteins using monoclonal and polyclonal antibodies to tropomyosin, α-actinin, vinculin, myosin, clathrin, calmodulin, brain spectrin (fodrin) and red cell spectrin. We found that Hirano bodies are entirely filamentous and do not contain granular structures. In addition to actin, we found that tropomyosin, α-actinin and vinculin epitopes are present in the Hirano bodies. All of these epitopes are uniformly distributed throughout the body. The actin in Hirano bodies is present in the F-form.

MATERIALS AND METHODS

Light Microscopy

Sections of hippocampus containing large numbers of Hirano bodies were prepared from tissue either fixed in 10% formalin in 0.1 M phosphate, pH 7.0, and embedded in paraffin, or frozen in isopentane chilled in liquid nitrogen.

Immunocytochemistry

Paraffin-embedded tissue was sectioned 8 μm thick and immunostained by the peroxidase–anti-peroxidase technique (29). Diaminobenzidine (DAB) (Sigma Chemicals, St. Louis, MO) (0.75 mg/ml) was used as cosubstrate with H2O2 (0.015%) in 0.05 M Tris-HCl, pH 7.6. Development times were generally less than two minutes (min). Sections were left unstained or were counterstained with hematoxylin and viewed at ×200 to ×1,000.

Antibodies

The following antibodies were used: A rabbit antiserum against chicken gizzard actin (AGA) and a mouse monoclonal antibody (C4) showing reactivity to all vertebrate isoactins (Dr. James Lessard, University of Cincinnati); rabbit antibodies to smooth muscle tropomyosin (Dr. Robert Goldman, Northwestern University); brain tropomyosin (Dr. Anthony Bretschger, Cornell University); human platelet and chicken skeletal muscle tropomyosin (Dr. Sarah Hitchcock-DeGregori, Rutgers University); chicken gizzard tropomyosin (Dr. Fumio Matsumura, Cold Spring Harbor Laboratories); α-actinin (Miles Laboratory), avian red blood cell spectrin (Ken Edds, State University of New York, Buffalo), brain spectrin (fodrin) (Dr. Mark

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Willard, Washington University) and squid brain myosin (Dr. Karl Fath, University of North Carolina); monoclonal antibodies to vinculin (Miles Laboratory), chicken skeletal muscle myosin (Dr. Donald Fischman, Cornell University Medical School), and clathrin (Dr. Frances Brodsky, Becton-Dickinson Laboratories); and a sheep antiserum to calmodulin (Dr. John Dedman, University of Texas Medical School, Houston). As controls, the sera were adsorbed with chicken gizzard actin or α-actinin (Sigma Chemical Co.), or rabbit skeletal muscle tropomyosin (purified according to the method of Bailey (30)) overnight at 4°C, then centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was used for immunocytochemistry. Also, non-immune and preimmune rabbit and mouse sera and normal goat serum as primary antibodies served as controls. Other controls consisted of antibodies absorbed with ovalbumin instead of actin, α-actinin or tropomyosin.

**Fluorescence Microscopy**

Sections were cut 8 μm thick from tissue frozen in liquid nitrogen in isopentane, then fixed with 10% buffered formalin, pH 7.0, for ten min. After a brief rinse with phosphate buffered saline (PBS), pH 7.4, they were permeabilized in acetone, five min at −20°C. Sections were stained with fluorescein- or rhodamine-conjugated phalloidin (Molecular Probes) (165 ng/ml in PBS) for 20 min; followed by one rinse in PBS. The slides were mounted in Aquamount (Lerner Laboratories) and examined under epi-illumination by using a Leitz Dialux equipped with 50 watt mercury illumination.

**Immunostaining for Electron Microscopy**

Tissue was fixed in 10% formalin in phosphate buffer, pH 7.0, and sectioned (60 μm) with a Vibratome (Oxford Instruments). The sections were stained by using the peroxidase-anti-peroxidase procedure (29) by using the primary antibodies, at the titer determined to be optimal for light microscopic immunostaining. After washing, the sections then were incubated with anti-mouse immunoglobulin (goat) or anti-rabbit immunoglobulin (goat) and then with mouse or rabbit peroxidase-anti-peroxidase complex. All antibody incubations were for at least 24 hours (h) at 4°C to ensure antibody penetration. The sections then were incubated with DAB 0.75 mg/ml in 0.015% H₂O₂ 0.05 M Tris-HCl at pH 7.6, for less than five min, conditions that minimize diffusion of oxidized DAB.

Immunogold staining was also performed. One set of 60 μm sections was treated with 0.1% Triton X-100 (Sigma) in Tris buffered saline (TBS) for 15 min at 20°C before incubation with the primary antibody for 24 h at 4°C. This was followed by a 48 h incubation at 4°C with colloidal gold (20 nm) which had been complexed to either affinity purified goat anti-rabbit (6) or anti-mouse IgG for 48 h at 4°C.

Sections prepared for the immunoperoxidase or immunogold were rinsed with 0.2 M cacodylate pH 7.4, treated with 1.0% OsO₄ in 0.2 M cacodylate at pH 7.4 for 45 min, dehydrated and embedded flat in Spurr’s medium. Thin sections stained with colloidal gold were contrasted with uranyl acetate and lead citrate; those immunostained by the peroxidase-anti-peroxidase technique were not. Sections were examined at 60 kV on a JEOL 100CX electron microscope.

**High Voltage Electron Microscopy**

Sections from non-immunostained tissue in Spurr’s medium were cut 1.0 μm and 0.5 μm thick, contrasted with uranyl acetate and lead citrate, and examined at one million electron volts at the Laboratory for High Voltage Electron Microscopy, University of Colorado, Boulder (Dr. Richard McIntosh, Director).

**Characterization of Antibody**

*Preparation of Tissue Samples for Electrophoresis:* Normal human grey and white matter homogenates were prepared after stripping meninges and blood vessels from frozen human brain tissue. The tissue (57 mg) was disrupted in a Dounce homogenizer (Kontes Glass Co.) using 11.4 ml chloroform-methanol, centrifuged at 40,000 rpm and the supernatant discarded.
The pellet was mixed 1:10 with 0.57 ml of a combination of 2% SDS, a 2 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM Tris-HCl, pH 6.8.

**SDS PAGE and Immunoblotting:** The system of Laemmli (31) was used. Molecular weight standards (Pharmacia Fine Chemicals, Piscataway, NJ) included thyroglobulin (330 kilodalton [kD]), ferritin (220 kD), albumin (67 kD), catalase (60 kD), lactate dehydrogenase (36 kD), and ferritin (18.5 kD). Immunoblotting was performed as previously described (5). Samples of brain white and grey matter, chicken gizzard actin, chicken gizzard α-actinin, and skeletal muscle tropomyosin were electrophoresed. Proteins were transferred onto nitrocellulose paper (Biorad) at 0.3 amperes for 18 h at 4°C. The blots were incubated in 10% NGS-TBS (37°C for one h). The strips were incubated with rabbit and mouse actin antibodies (1:100 in 1% NGS-TBS) adsorbed with chicken gizzard actin, and rabbit smooth muscle tropomyosin antibodies (1:100 in 1% NGS-TBS) adsorbed with skeletal muscle tropomyosin and rabbit α-actinin antibody (1:100 in 1% NGS-TBS) adsorbed with chicken gizzard α-actinin, and with the corresponding unadsorbed antibodies, and with anti-platelet tropomyosin (1:200 in 1% NGS-TBS). The paper strips were incubated with primary antibodies for two h at room temperature. They were rinsed with 0.05% Tween 20 in TBS at room temperature, then were incubated with secondary antibody–peroxidase conjugate (1:1,000 in 1% NGS-TBS) (Cappel Laboratories) for one h at room temperature. They were rinsed in 0.05% Tween 20 in TBS, then in 0.05 M Tris-HCl at pH 7.6 twice for ten min each, followed by development in DAB (0.75 mg/ml in 0.05 M Tris-HCl at pH 7.6) for less than five min.

**RESULTS**

**Actin**

*Light Microscopy:* Both the actin antibodies stained the Hirano body. The staining was throughout the structure. Optimal staining was obtained for the rabbit actin antibody, at 200 μg IgG fraction/ml (1:50) and at 10 μg protein/ml for the mouse monoclonal antibody to actin (1:100). No staining of Hirano bodies was observed using normal rabbit or mouse sera. Hirano body staining by the mouse and rabbit actin antibodies was eliminated by adsorption with 250 μg/ml and 15 μg/ml chicken
gizzard actin, respectively. Staining was not affected by adsorption with 1 mg/ml ovalbumin.

Electron Microscopy: The rabbit anti-actin serum stained Hirano bodies using both immunoperoxidase and colloidal gold. Gold decoration appears to be primarily distributed in cross-hatched areas (Fig. 1) rather than in areas with a "bead-on-a-string" arrangement. This may reflect differences in accessibilities of the two configurations, or be an artifact of the greater area examined in the cross-hatched areas. The mouse monoclonal antibody strongly stained Hirano bodies by using the immunoperoxidase technique (Figs. 2, 3). The staining for both antibodies was diffuse throughout the Hirano body. The controls for both immunogold (Fig. 4) and immunoperoxidase (Fig. 5) were unstained.

Fluorescence Microscopy: Both rhodamine (Fig. 6) and fluorescein conjugated phalloidin stained Hirano bodies.

Antibody Characterization: Immunostaining on blots of chicken gizzard actin and human brain white matter by both the monoclonal actin (Fig. 7a) and polyclonal (Fig. 7c) antibodies demonstrated reactivity with a 43 kD molecular weight band. Staining is blocked by adsorption with 15 (Fig. 7b) and 250 μg/ml (Fig. 7d) chicken gizzard actin, respectively.

Tropomyosin

Light Microscopy: The smooth muscle and platelet tropomyosin antibodies stained Hirano bodies (Fig. 8a). The staining was also seen diffusely throughout the Hirano

body. Optimal staining was with anti-smooth muscle tropomyosins, 1:100, and anti-platelet tropomyosin, 1:200. No staining of Hirano bodies was observed using rabbit non-immune or pre-immune sera. Staining by each antibody was removed by adsorption with 125 µg/ml rabbit skeletal muscle tropomyosin (Fig. 8b). Staining was not blocked by adsorption with 1 mg/ml ovalbumin.

Electron Microscopy: The smooth muscle tropomyosin antibody, which was used for both immunoperoxidase and colloidal gold, stained the Hirano bodies. The anti-platelet tropomyosin, which was used for immunoperoxidase, yielded a diffuse intense staining of Hirano bodies (Fig. 9a). The reaction product was distributed around both cross-hatched and “bead-on-a-string” arrangement of filaments (Fig. 9b). Gold decoration was not as intense as for the anti-actin. The controls were unstained (Fig. 9c) or lacked gold decoration.

Antibody Characterization: On blots of human white matter, using smooth muscle tropomyosin antibody, bands were stained corresponding to 30 and 32 kD proteins, whose intensity was diminished by adsorption with 500 µg/ml rabbit skeletal muscle tropomyosin (Fig. 10a, b). The platelet tropomyosin antibodies each stained a band on a blot of human white matter corresponding to a 32 kD protein (Fig. 10c). Staining of blots of the rabbit skeletal muscle tropomyosin (used for adsorption) with the smooth muscle tropomyosin antibody showed bands corresponding to 30 kD and 32 kD proteins (Fig. 11a), which was blocked by adsorption with 500 µg/ml rabbit skeletal muscle tropomyosin (Fig. 11b).

α-Actinin

Light Microscopy: The α-actinin antibody stained Hirano bodies (Fig. 12a); staining was optimal at 1:100. No staining was seen with non-immune serum, or after
Fig. 6. Sommer's sector of hippocampus stained with rhodamine phalloidin showing staining of a Hirano body (arrow). ×875.

adsorption with 500 μg/ml of chicken gizzard α-actinin (Fig. 12b). Staining was not blocked by adsorption with 1 mg/ml ovalbumin.

Antibody Characterization: When the α-actinin was electrophoresed by SDS-PAGE, the α-actinin antibody stained a 105 kD band on the blot (Fig. 13a), whose staining was blocked by adsorption with 1,000 μg/ml α-actinin (Fig. 13b).

Vinculin

Light Microscopy: The vinculin monoclonal antibody stained Hirano bodies; staining was optimal at 1:200. No staining was seen with mouse non-immune serum.

Other Antibodies

Light Microscopy: No staining of Hirano bodies was seen with antisera to brain or skeletal muscle tropomyosin, clathrin, calmodulin, myosin, red cell spectrin, or brain spectrin (fodrin).

Antibody Characterization: The antibody to squid brain myosin stained a 200 kD protein on one- and two-dimensional immunoblots of myosin and crude brain preparations (Karl Fath, unpublished results). The other antibodies have been characterized as previously described (32–38).

High Voltage Electron Microscopy

High voltage electron microscopy on 1.0 and 0.5 micron sections showed that the "beads" in the "bead on a string" arrangement were transformed into filaments when viewed at the proper orientation (Fig. 14).

DISCUSSION

The present study confirms the presence of actin epitopes in Hirano bodies and shows that the actin epitopes are diffusely distributed throughout these structures. In the previous light microscopic immunocytochemical study, actin epitopes appeared to be condensed at the periphery of Hirano bodies (11). The present ultrastructural study shows cracks and grooves in the Hirano bodies. These areas might have been seen as unstained areas at light microscopic examination and might have given a false impression of more intense staining at the intact edge of Hirano bodies.
Additional evidence supporting the presence of actin in the Hirano body is provided by their intense reaction with rhodamine and fluorescein phalloidin, probes having a high affinity for F-actin (28). The presence of polymerized actin suggests that in Hirano bodies actin is in a filamentous form and not as actin monomers interspersed with other proteins. The similarity in size between filaments of Hirano bodies and normal actin microfilaments is consistent with this possibility (23).

The results of our high voltage electron microscopy also confirm the filamentous nature of Hirano bodies. The section thickness in our study affords a greater representation of the change from beads to filaments for a given angle of rotation. Because the beads in the "bead-on-a-string" arrangement were visualized as filaments after tilting 1.0 μm and 0.5 μm sections, this finding indicates that Hirano bodies are made entirely of superimposed filaments, and do not contain granular structures. It can be seen that the beads are in fact cross sections of filaments rather than granules.

The main finding of this study is that tropomyosin, α-actinin and vinculin epitopes are also present in Hirano bodies. As with actin epitopes, they are uniformly distributed throughout the body. It has been previously hypothesized that tropomyosin is the major component of Hirano bodies, based on the ultrastructural resemblance of Hirano bodies to tropomyosin crystals (39). Although our study demonstrates the presence of tropomyosin, results of the immunogold decoration tend to suggest it is not the major component. Another possibility is that tropomyosin epitopes in the Hirano body are not as accessible to the tropomyosin antibody as are actin epitopes to the actin antibody.

The antibodies to tropomyosin reacting with Hirano bodies were raised to platelet and smooth muscle tropomyosin, while no reaction was seen with an antibody to brain or skeletal muscle tropomyosin. This finding has at least two explanations. First, we used only one antibody to brain tropomyosin; use of an additional anti-brain tropomyosin may yield staining. Second, since tropomyosin in brain is present in many structures other than neurons, the antibody we used might have been raised to a non-neuronal tropomyosin. This is likely because immunostaining of sections of hippocampus using the brain tropomyosin antibody was virtually confined to blood vessels and cell nuclei. It is not surprising that antibodies to non-neuronal tropomyosin stained Hirano bodies, because there are many isoforms of tropomyosin (40–43) with different actin binding properties (44). Different tropomyosin isoforms

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**Fig. 8.** Immunoperoxidase stained Hirano bodies in Sommer's sector of the hippocampus. 

b. (arrow) after adsorption with skeletal muscle tropomyosin. × 860.
Fig. 9. Diffuse staining throughout the Hirano body using the peroxidase–anti-peroxidase technique. a. The anti-platelet tropomyosin (1:200). ×11,600. b. At a higher power the reaction product can be seen distributed around filaments arranged both in the cross-hatched fashion (around asterisk) and in the "bead-on-a-string" fashion (arrow). ×22,000. c. The Hirano bodies treated with rabbit pre-immune serum (1:200) were unstained. ×11,200.
might be expressed in aging neurons or in neurons forming Hirano bodies; the brain tropomyosin antiserum may not recognize one of these isoforms. Evidence indicates that there may be genetic co-expression of more than one type of tropomyosin within a single cell (40). It is possible that genetic expression of a tropomyosin with a higher than normal actin binding affinity may occur in aging neurons; this higher affinity may initiate Hirano body formation.

The presence of tropomyosin, α-actinin and vinculin epitopes in the Hirano bodies is not surprising. If Hirano bodies result from an alteration of microfilaments, epitopes of proteins intimately associated with microfilaments also may be present. In non-muscle cells, cytoskeletal tropomyosin is bound to F-actin and may serve to stabilize the filaments (45–48) or protect them from actin-severing proteins (49–52). The latter characteristic may be particularly important in Hirano body formation, since actin-severing proteins may be unable to gain access to actin filaments due to “overprotection” by tropomyosin. The roles of α-actinin and vinculin are that of
Fig. 10. Human white matter electrophoresed on SDS-PAGE (molecular weight standards in thousands) anti-smooth muscle tropomyosin (1:100) before (A) and after (B) adsorption with skeletal muscle tropomyosin, and (C) with anti-platelet tropomyosin. 1:200.

Fig. 11. Skeletal muscle tropomyosin electrophoresed on SDS-PAGE (molecular weight in thousands); immunoblots with anti-smooth muscle tropomyosin (1:100) before (A) and after (B) adsorption with 500 g/ml skeletal muscle tropomyosin.

Fig. 13. Chicken gizzard α-actinin electrophoresed on SDS-PAGE (molecular weight in thousands). A. Immunoblots with anti-α-actinin (1:100). B. Immunoblot after adsorption with 1,000 μg/ml α-actinin.

cross-linking proteins (27, 53); thus cross-linking of actin filaments also may be related to Hirano body formation. Although no epitopes of other actin-associated proteins were shown by immunostaining, this negative finding does not exclude their presence. The antibodies to myosin, clathrin, calmodulin and spectrin may be directed to epitopes of these proteins that have become inaccessible or have been lost during the formation of the Hirano bodies.

The presence of the cytoskeletal proteins tropomyosin, α-actinin and vinculin provides additional evidence that Hirano bodies result from an alteration of the
neuronal cytoskeleton and underscores once again the role of the cytoskeleton in neuronal aging. It has been suggested that the neuronal cytoskeleton has two components, based on the findings that the three main elements of the cytoskeleton, i.e., neurofilaments, microtubules and microfilaments, do not always codistribute within the neuron, and that the proteins corresponding to these elements are not transported at the same rate in the axon (54). One component is the neurofilament and microtubule network, in which neurofilaments and microtubules are connected by the heavy neurofilament subunits, microtubule-associated proteins, and perhaps other proteins as side arms (55). The second component is a system of microfilaments made of actin and actin-associated proteins (54, 56–58).

Paired helical filaments, the main component of the neurofibrillary tangles of Alzheimer type, are quite different from the straight filaments which characterize the Pick bodies of Pick's disease or the neurofibrillary inclusions of progressive supranuclear palsy (59–68). Despite these structural differences, the fibrillary components of these three inclusions all share the same known epitopes which normal neurofilaments and microtubule-associated proteins (5, 68–72). Hirano bodies do not immunoreact with antibodies to neurofilaments (3). Thus, Hirano bodies are likely to result from alterations of cytoskeletal components different at least in part from those involved in the formation of paired helical filaments, the straight filaments of Pick bodies and the inclusions of progressive supranuclear palsy. A hypothesis that deserves further study is that while paired helical filaments derive largely from a derangement of the neurofilament-microtubule system, Hirano bodies result predominantly from an alteration of the microfilament system.

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Fig. 14. A 0.5 μm plastic embedded section examined at one million electron volts. (a) is positioned at \(-10^\circ\) and (b) at \(+10^\circ\). Note change of "beads" in the bead-on-a-string arrangement in (a), to filaments in (b). ×51,300.

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